DOI:
10.1002/JLB.5MA1219-296R

Document Version
Peer reviewed version

Pitfalls in the characterization of circulating and tissue-resident human γδ T cells

Nicola Beucke[*], Daniela Wesch[†], Hans-Heinrich Oberg[†], Christian Peters[†], Jonas Bochem[*], Benjamin Weide[*], Claus Garbe[*], Graham Pawelec[‡,§], Susanne Sebens[¶], Christoph Röcken[||], Hisayoshi Hashimoto[#], Markus W. Löffler[‡,**,††], Paola Nocerino[‡‡], Shahram Kordasti[‡‡], Dieter Kabelitz[†], Karin Schilbach[#], Kilian Wistuba-Hamprecht[*]

[*] Department of Dermatology, University Medical Center, Tübingen, Germany

[†] Institute of Immunology, Christian-Albrechts University of Kiel, Kiel, Germany

[‡] Interfaculty Institute for Cell Biology, Department of Immunology, University of Tübingen, Tübingen, Germany

[§] Health Sciences North Research Institute, Sudbury, ON, Canada

[¶] Institute for Experimental Cancer Research, Christian-Albrechts-University of Kiel, Kiel, Germany

[||] Institute of Pathology, University Hospital Schleswig-Holstein, Kiel, Germany

[#] Department of Pediatric Hematology and Oncology, University Children's Hospital Tübingen, Tübingen, Germany

** Department of General, Visceral and Transplant Surgery, University Hospital Tübingen, Tübingen, Germany.

[††] Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany

[‡‡] Systems Cancer Immunology, Comprehensive Cancer Centre, King's College London, London, UK

Correspondence: Kilian Wistuba-Hamprecht, Department of Dermatology, University Medical Center, Waldhörnlestraße 22, 72072 Tübingen, Germany. Email: kilian.wistuba-hamprecht@uni-tuebingen.de

Short running title: Pitfalls in the characterization of γδ T cells

Summary sentence: Overviewing the state-of-the-art and own experiences, highlighting pitfalls with commercially-available reagents for γδ T cell characterization by flow cytometry, CyTOF, magnetic cell isolation or immunohistochemistry.

Keywords: Flow Cytometry, Mass Cytometry, Immunohistochemistry, Magnetic Cell Isolation, Immunomonitoring

Abbreviations

ADCC: Antibody-dependent cellular cytotoxicity

AEC: Aminoethyl-carbazole

APC: Allophycocyanine

BV: Brilliant Violet

CMV: Cytomegalovirus

CyTOF: Mass cytometry

EBV: Epstein-Barr-Virus

EDTA: Ethylenediaminetetraacetic acid

EPCR: Endothelial protein C receptor

Er: Erbium

FITC: Fluorescein isiothiocyanate

Gd: Gadolinium

IHC: Immunohistochemistry

Ir: Iridium

Monoclonal antibody: mAb

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate-buffered saline

PE: Phycoerythrin

PHA-L: Phytohemagglutinin-L

PI: Positive isolation

PMA: Phorbol 12-myristate 13-acetate

Pr: Praseodymium

Sm: Samarium

TCR: T-cell receptor

Abstract

Dissection of the role and function of human γδ T cells and their heterogeneous subsets in cancer, inflammation and auto-immune diseases is a growing and dynamic research field of increasing interest to the scientific community. Therefore, harmonization and standardization of techniques for the characterization of peripheral and tissue-resident γδ T cells is crucial to facilitate comparability between published and emerging research. The application of commercially-available reagents to classify γδ T cells, in particular the combination of multiple antibodies, is not always trouble-free, posing major demands on researchers entering this field. Occasionally, even entire γδ T cell subsets may remain undetected when certain antibodies are combined in flow cytometric analysis with multicolor antibody panels, or might be lost during cell isolation procedures. Here, based on the recent literature and our own experience, we provide an overview of methods commonly employed for the phenotypic and functional characterization of human γδ T cells including advanced polychromatic flow, mass cytometry, immunohistochemistry and magnetic cell isolation. We highlight potential pitfalls and discuss how to circumvent these obstacles.

Introduction

Knowledge of the orchestration of γδ T cells in the ensemble of immunity is still limited, especially in humans. These “unconventional” T cells are a numerically minor population in peripheral blood, representing 1-10% of all T cells and are, unlike αβ T cells, not MHC-restricted [1]. Knowledge of γδ T cell receptor (TCR) ligands is sparse; only a few, structurally diverse molecules such as phosphoantigens, CD1, endothelial protein C receptor (EPCR) and other cell-surface structures have been identified [2], suggesting an immense potential for diversity. The γδ T cell population comprises heterogeneous subsets with various functions including secretion of cytokines such as TNF, IFN-γ and IL-17 [3], cytotoxic activity via the granzyme-perforin axis and antibody-dependent cellular cytotoxicity (ADCC) by CD16 expressing cells [4], antigen-presentation functions [5] and interactions with B cells promoting immunoglobulin class switching [6]. Vδ1 γδ T cells are the predominant T cell subset in some tissues, accounting for around 40% of all intra-epithelial lymphocytes in the large intestine, for example [7]. The Vδ1 TCR repertoire is often private, highly focused on a few clones, and displays features of the adaptive immune system [8]. On the other hand, Vδ2 γδ T cells, which dominate the γδ TCR repertoire in peripheral blood, have a semi-invariant TCR, a diverse public repertoire and mainly behave in an innate-like manner [9]. Due to their pleiotropic roles in immunity and implications in cancer, infectious and auto-immune diseases, γδ T cells are of rapidly growing interest. Transcriptomic analyses have documented that intra-tumoral γδ T cells may represent significant favorable prognostic immune cell populations in several different cancers [10] and Oberg et al. observed large numbers of different γδ T cell subsets infiltrating isolated pancreatic and ovarian tumors *ex vivo* [11, 12, Oberg et al. this volume]. In areas other than cancer, γδ T cells have been shown to

contribute to the immune response against CMV [13, 14] and malaria [15], and to be involved in various inflammatory conditions [16]. It is therefore important to harmonize and standardize techniques for the investigation of γδ T cells in order to avoid potential pitfalls when using and combining commercially-available antibodies and comparing results between centers. Here, we aim to provide a basic framework for the phenotypic and functional characterization of peripheral and tissue-resident γδ T cells, including magnetic cell isolation, advanced polychromatic flow cytometry, mass cytometry (CyTOF) and immunohistochemistry (IHC).

Material and Methods

Study participants and sample acquisition

Blood samples were obtained from healthy adult volunteers at the Department of Hematology and Oncology, Children's Hospital, University of Tübingen (Project no. 38/2009BO2, 470/2013BO2, 673/2015BO2, 105/2017BO2 and 880/2017BO2) and from the biobank at the Interfaculty Institute of Cell Biology (IFIZ), Department of immunology, University of Tübingen (Project no. 156/2012BO1 and 633/2019BO2). Tissue samples from patients with EBV-associated Hodgkin's lymphoma (with pathological features assessed according to the WHO classification) and colon carcinoma (staged according to the UICC TNM classification system) were obtained at the University Hospital Schleswig-Holstein, Kiel (D430/09). Written informed consent was obtained from all blood and tissue donors. This study was conducted in accordance with the Declaration of Helsinki and applicable laws and regulations, and has been approved by the respective institutional review boards (Ethics Committees at the University Hospital Schleswig-Holstein in Kiel and at the University Hospital Tübingen).

Magnetic cell isolation

γδ T cells were isolated from fresh peripheral blood mononuclear cells (PBMCs) via magnetic cell isolation using the following commercial kits: i) Immunomagnetic negative selection cell isolation kit: TCRγ/δ+ T Cell Isolation Kit Human (Miltenyi Biotec) or Immunomagnetic negative selection cell isolation kit: EasySep™ Human Gamma/Delta T Cell Isolation Kit (STEMCELL Technologies) or, as we show in Figure 1, customized versions of both kits were used, both omitting anti-CD16 antibodies. Immunomagnetic

positive selection was performed with a fluorochrome-labelled Vδ2-specific antibody (clone B6, BD) and for secondary labelling and anti-fluorochrome sorting, either anti-FITC Micro-beads (Miltenyi Biotec) or anti-PE MicroBeads Ultra Pure (Miltenyi Biotec) or an Immunomagnetic positive selection kit EasySep™ PE Positive Selection Kit II (STEMCELL Technologies) were used.

Polychromatic flow cytometry

Phenotypic and functional analysis followed standardized protocols on cryopreserved samples. For immunomonitoring studies, cryopreservation is still the gold standard to minimize bias introduced by batch to batch variance in polychromatic flow cytometry, although the expression pattern of some particular markers may be affected. In brief, peripheral blood was drawn and anticoagulated using EDTA, followed by Ficoll-Hypaque density gradient centrifugation to isolate PBMCs. After washing twice, the cells were cryopreserved in medium with 10% DMSO and 20% FCS in RPMI-1640 and stored at -196^{o}C. Cryopreserved PBMCs were thawed, incubated with an Fc-receptor-blocking reagent (Gammunex, Grifols) and ethidium monoazide (EMA, Biotium) or LIVE/DEAD fixable red (Thermo Fisher Scientific) to label dead cells. Next, characteristic cell surface antigens of γδ T cells were stained using the following monoclonal antibodies (mAbs) to illustrate various common panel compositions: CD3 Alexa Fluor 700 and CD3 BV510 (both clone UCHT1, Biolegend), pan-γδ TCR Biotin (clone 11F2, Miltenyi Biotec), pan-γδ TCR FITC (clone 11F2, BD), pan-γδ TCR purified and pan-γδ TCR PE (both clone IMMU510, Beckman Coulter), Vδ1 TCR FITC, Vδ1 TCR PerCP-Vio700 and Vδ1 TCR APC (all clone REA173, Miltenyi Biotec), Vδ2 TCR PerCP (clone B6, Biolegend), Vδ2

TCR PE and Vδ2 TCR FITC (both clone 123R3, Miltenyi Biotec), Streptavidin-PE (Biolegend), F(ab')2-Fragment goat anti mouse Pacific Orange (Invitrogen). For characterization of established T cell clones, antibodies against Vγ2/3/4 (clone 23D12) [17], Vγ3/5 (clone 56.3) [18] and pan-αβ TCR FITC (clone IP26, Biolegend) were used. The generation of 56.3^+ T cell clones has been described previously. In brief, 56.3 positive cells were selected by MACS from PBMCs from healthy donors and were cloned at 0.3 cells per well in the presence of irradiated feeder cells, PHA 0.5 μg/mL and IL-2 (50 IU) [18].

For intracellular staining, cryopreserved PBMCs that had been stimulated with PMA (20 ng/ml, Sigma) and Ionomycin (750 ng/ml, Merck) or Zoledronate (5 μM, Hexal) and incubated with Brefeldin A (GolgiPlug, BD), Monensin (GolgiStop, BD) and CD107a Pacific Blue (clone H4A3, Biolegend) for 12h, were fixed and permeabilized using a fixation/permeabilization solution kit (BD) and stained with the following antibodies: IFN-γ PE-Cy7 (clone B27, Biolegend), IL-17A BV711 (clone BL168, Biolegend), TNF-α Alexa Fluor 700 (clone Mab11, Biolegend). Proliferation in response to stimulation with PHA-L (Roche) was tracked by labelling cells with CellTraceViolet (Thermo Fisher Scientific). Optimal results were achieved when seeding $0.2x10^6$ cells in 200 μL medium per well in 96-well U bottom plates (Greiner). Proportional upscaling, e.g. seeding $0.6x10^6$ cells in 600μl medium in a 48-well plate, was possible though not optimal, enabling analysis of donors with low percentages of γδ T cells, patient samples and subpopulations. Panels for functional analysis and for tracking of proliferation are summarized in Table 1. Data were acquired using a three laser LSR II (BD) with FACSDiva software V6.1.3 (BD) and customized filter settings and data analysis was performed with FlowJo V10.5.3 (BD; gating strategy, Supplementary Figure 1).

Mass cytometry

Mass cytometric analysis followed established protocols [19]. In brief, cryopreserved PBMCs were thawed, dead cells were stained with Rhodium 103 and cell surface Fc receptors were blocked. Samples were then incubated with an antibody cocktail for cell surface staining which contained antibodies against CD3 170Er (clone UCTH1, Fluidigm), pan-γδ TCR 152Sm (clone 11F2, Fluidigm), Vδ2 TCR141Pr (clone B6, Biolegend; in house-conjugated, using Fluidigm´s Maxpar antibody labelling kit; catalog no.: 201141B) and Vδ1 TCRFITC (clone TS8.2, Thermo Fisher Scientific) amongst others. Next, an anti-FITC 160Gd antibody (Fluidigm) was used to stain Vδ1 T cells. After fixation, permeabilization and staining of intracellular antigens, samples were incubated for at least 12 hours in a solution of 4% paraformaldehyde in PBS. Samples were stained in batches with 125Ir on the mornings of the respective days of data acquisition. Each sample was rebuffered in purified water directly before acquisition on a Helios system (Fluidigm) at King´s College London. Data analysis was performed with FlowJo V10.5.3 (BD).

Immunohistochemistry

Immunostaining with mAbs against γδ TCR (clone γ3.20, Thermo Fisher Scientific), Vγ9 TCR (clone 7A5) [20], Vγ2/3/4 TCR (clone 23D12) [17, 18] or mouse IgG1 isotype control (Thermo Fisher) of serial paraffin-embedded tissue sections from patients with EBV-associated Hodgkin's lymphoma was carried out after deparaffinization with the fully automated Bond Max-system using the Bond Polymer Refine Detection Kit (Leica-Menarini). Automated antigen retrieval was performed in in Bond Epitope Retrieval

206 Solution 1 (citrate buffer pH 6.0; Leica-Menarini). Additionally, immunostaining with
207 Vγ2/3/4 TCR (clone 23D12), Vδ1 TCR (clone R3.12, Beckman Coulter) or mouse IgG1
208 isotype control of cryopreserved sections from patients with colon carcinoma was done
209 after acetone fixation and blockade with 4% bovine serum albumin. As second step,
210 antibody EnVision mouse horseradish peroxidase (DAKO) was used. The substrate
211 reaction was performed using the AEC substrate for peroxidase (DAKO). Finally,
212 sections were stained with hematoxylin and embedded in glycerine gelatine (Merck).

Results and Discussion

Magnetic isolation of γδ T cells and subsets

Isolation of γδ T cells is a critical procedure and the methodology of choice needs to be adapted to the design of each particular experiment. The total γδ T cell compartment can be positively or negatively selected using kits from commercial suppliers (e.g. Miltenyi Biotec and STEMCELL Technologies) (Figure 1A, 1B). In addition to beads directly coupled to a γδ TCR-targeting antibody (Miltenyi Biotec), secondary labelling strategies allow sorting and discriminating γδ T cell subsets. Multi-sort beads facilitate multiple rounds of positive selection because these labels can be removed. Positive isolation (PI) is suitable for separating aminobisphosphonate-expanded γδ T cells or γδ T cells expressing particular Vγ/Vδ elements from PBMCs (Figure 1B), and γδ T cells from intra-tumoral lymphoid compartments, e.g. for TCR sequence analysis, offering the advantage of sparing other (infiltrating) cells for further isolation/analysis. On the other hand, for studies examining cell activation status, receptor signaling, cytokine expression and/or cytotoxicity, γδ T cells should be negatively selected to avoid antibody-cross-linking of the γδ TCR. Although some manufacturers claim that immune cells experience no activation through their PI procedure, γδ T cells do upregulate CD69 after PI [21] and show significant functional bias compared to negatively selected cells from the same donor. The EasySep™ Human Gamma/Delta T Cell Isolation Kit from STEMCELL Technologies removes non-γδ T cells with tetrameric antibody complexes and dextran-coated magnetic particles by retaining them inside a tube using a strong magnetic field. Whereas labelled cells remain attached to the tube wall, the remaining (negatively-selected) cells can be poured into a separate tube. Similarly, Miltenyi

Biotec's biotin-conjugated depletion cocktail eliminates non-γδ T cells via a secondary magnetic label that retains them on a "MACS® Column" in a magnetic field. High purities – above 98.5% γδ T cells – are routinely achieved with both methods independent of the initial content of γδ T cells and obtained isolates do not contain any undesired αβ T cells (Fig. 1A). The "untouched" negatively-sorted cells are not activated. The STEMCELL Technologies procedure is faster and yields remain quantitative, even when drastically downscaling initial cell numbers (due to zero dead space volume). Moreover, physiological stressors such as mechanical stress, centrifugal forces, and extensive incubation at unphysiologically low temperatures during labelling and purification are avoided.

Pitfalls that may be encountered, when using either of these negative selection procedures are: i) depleted cells are heavily labelled and can not be used for further downstream applications; ii) depletion cocktails often contain antibodies targeting molecules also expressed by subpopulations of γδ T cells, e.g. CD16, which may severely bias subsequent (functional) studies, such as ADCC or gene expression analyses. We therefore suggest the use of anti-NKp46 (a lineage marker of NK cells) and anti-NKp30 instead of anti-CD16 for NK-cell elimination during negative γδ T cell isolation. NKp46 and NKp30 are expressed on peripheral NK cells but not on γδ T cells in the peripheral blood. However, it should be noted that long-term activation of γδ T cells induces the expression of NKp46, NKp30 and also of Nkp44 in certain subsets (Supplementary Figure 2) [22]. Until commercial kits that exclude anti-human CD16 mAbs become available customized kits are an option.

When aiming to negatively isolate specific subsets of γδ T cells, a combination of strategies is required. For selecting untouched Vδ2 γδ T cells from PBMCs, Vδ1 and

other γδ T cell subsets such as Vδ3 γδ T cells must be removed (via PI) before γδ T cell negative selection yields the Vδ2 T cell subset isolate. Unless an anti-human Vδ3 mAb is available, individuals with high numbers of $V\delta1^{-}V\delta2^{-}$ γδ T cells are not suitable for Vδ2 negative selection (Figure 1C). A potential candidate for this approach might be the monoclonal anti-human T cell receptor Vδ3 antibody (clone P11.5B), which was previously distributed by Gentaur and Coulter. However, at the time of writing, the commercial availability of this antibody, its format and thus its suitability for magnetic cell isolation remain unclear.

Phenotypic and functional analysis via polychromatic flow cytometry

When designing an antibody panel for multicolor flow cytometry a few considerations need to be taken into account, in order to avoid certain γδ T cell subsets remaining undetected, and thus biasing subsequent analysis. Below, we aim to highlight the commonest problems and to provide a framework for the flow cytometric analysis of γδ T cells. Furthermore, we briefly present panels designed for the investigation of phenotypic markers, as well as functional and proliferative properties.

We previously reported that the unconjugated pan-γδ TCR antibody clone 11F2 was the only tested antibody able to detect all γδ T cells when combined with a Vδ2 antibody (B6, IMMU389), while the conjugated forms of the commercially-available clones 11F2, B1/B1.1 do not always stain 100% of the γδ T cell population [23]. Moreover, clone B1/B1.1 is unsuitable for multicolor flow cytometry panels including a CD3 antibody due to interference between these two antibodies [23]. The recently-developed generation of γδ TCR subset-specific antibodies, namely Vδ1 clone REA173 and Vδ2 clone 123R3 in combination with pan-γδ TCR antibodies (11F2, IMMU510) seems to offer a standard for

flow cytometric characterization of γδ T cells (Figure 2A), overcoming the above-mentioned issues (Supplementary Figure 3A). We tested directly fluorophore-labelled and secondarily detected pan-γδ TCR antibodies (11F2 and IMMU510) to achieve an optimal balance between a rapid and straightforward staining protocol, low background signal and a high staining index (Figure 2B). Separation of the γδ T cell population via directly-labelled pan-γδ TCR antibodies (11F2, IMMU510) can be problematic in the detection of Vδ1 T cells with low surface TCR expression levels (Supplementary Figure 3B) as seen in patient samples or in *in vitro* culture systems. Detection of the purified formats of these antibodies via fluorophore-labelled anti-mouse antibodies is generally not preferable due to prolonged staining procedures and high background signals. We identified the biotinylated 11F2 clone as an optimal choice (Figure 2A, 2B), because the biotin/streptavidin detection system combines signal amplification with simultaneously low background, resulting in improved separation of the target population, especially in fixed cells (Supplementary Figure 3C). The prolonged staining protocol (1) pan-γδ TCR, (2) streptavidin conjugate, (3) surface antibody cocktail can be reduced by integration of the streptavidin conjugate into the antibody master mix for extracellular staining. In general, use of PE-conjugates for pan γδ T cell antibodies is recommended, because PE itself is recognized by certain γδ TCRs and thus stains a small percentage of peripheral γδ T cells [24]. This should also be kept in mind when tandem conjugates containing PE (e.g. PE-Cy7, PE-Cy5.5 etc.) are included in the antibody panel. As stated above, the Vδ2 123R3 antibody is, in contrast to clone B6, compatible with fluorophore-conjugated pan-γδ TCR antibodies. Of note, clone B6 might be specific for the Vγ9Vδ2 TCR pairing, because it has been reported that detection of the $Vγ9^{-}Vδ2^{+}$ population is

not possible with clone B6, but clone 123R3 can be used as an alternative to also detect the rare population of $V\gamma9^{-}V\delta2^{+}$ T cells [9].

On the basis of this framework for detection of γδ T cells, we developed several polychromatic antibody panels: i) a phenotypic γδ T cell panel including markers for differentiation state [23] ii) a panel to monitor functionality of γδ T cells including the degranulation marker CD107a and the cytokines TNF, IFN-γ and IL-17A (Figure 3A) and iii) a panel to track γδ T cell proliferative capacity using CellTraceViolet (Figure 3B). The above-described basic phenotypic markers (pan-γδ TCR, Vδ1 and Vδ2) also worked well after fixation and permeabilization.

Using a combination of available mAbs, flow cytometry is also useful to monitor the entire expressed human Vγ repertoire [18]. Such an analysis is based on antibodies detecting Vγ9 (e.g., clone 7A5) [20], Vγ2/3/4 (clone 23D12) [17, 25], Vγ3/5 (clone 56.3) [18] and Vγ8 (clone R4.5.1) [26, 27]. As an example, the combination of mAb 56.3 and 23D12 unequivocally identifies γδ T cell clones expressing Vγ3 ($56.3^{+}23D12^{+}$) and Vγ5 ($56.3^{+}23D12^{-}$) (Figure 4A, 4B). Moreover, such antibodies are useful for detecting rare αβ T cells with a trans-rearranged TCR [13, 28]. As shown in Figure 4C, the 56.3-positive clone (established by positive selection of 56.3-positive cells from PBMC) stains with a pan-αβ T cell antibody (clone IP26) but not with a pan-γδ antibody (clone 11F2). These cells carry an in-frame Vγ5-Jβ-Cβ trans-rearrangement [18] .

Identification of immune signatures via mass cytometry

A good choice of markers for basic identification of cell populations intended to be divided into numerous subsets is essential for mass cytometric analysis, as currently up

to 40 channels can be acquired in parallel and multidimensional, automated data analysis is performed. At the time of writing, the only commercially-available pan γδ T cell antibody suitable for mass cytometry derives from the 11F2 clone. We identified even greater problems in mass cytometry than those we faced in polychromatic flow cytometry when using the 11F2 TCR γδ 152 Sm antibody in combination with custom-made B6 TCR Vδ2 141 Pr and TS8.2 Vδ1 FITC antibodies (detected via an anti-FITC 160 Gd). Large proportions of Vδ1 T cells were stained with the pan-γδ TCR antibody, but none or only a fraction of the Vδ2 T cells (Figure 2C). Steric hindrance caused by close proximity of the recognized epitopes and size and nature of the antibody tags might account for these observations. Further testing of the combinations of Vδ1 REA173 and Vδ2 123R3 with the pan-γδ 11F2 antibodies that achieved good resolution in polychromatic flow cytometry is also warranted in mass cytometry.

Immunohistochemical detection of tissue-associated γδ T cells

IHC-based tissue analysis enables tissue-infiltrating (γδ) T cell subsets to be analyzed in the context of their native surroundings, thereby providing a complementary approach to the above-discussed flow cytometry experiments. In the cancer setting, monitoring the abundance of tumor-infiltrating γδ T cells and the localization of distinct γδ T cell subsets can provide a more comprehensive assessment of the tumor status [11]. The best choice for analysis of the γδ TCR expression was the anti-TCR γ clone γ3.20. As we previously reported, IHC staining of consecutive paraffin-embedded sections of pancreatic ductal adenocarcinoma tissue revealed that a large proportion of the $CD3^+CD8^+$ T cells in the ductal epithelium were γδ T cells [11, 12]. Unfortunately the

clone γ3.20 is no longer available, but Jungbluth et al. recently reported that the TCR δ antibody clone H-41 (SC-100289, Santa Cruz) is an alternative for the detection of γδ T cells in paraffin-embedded tissue [29]. To visualize the distribution of different γδ T cell-subsets, serial tissue sections were stained with our in-house Vγ9 and Vγ2/3/4 antibodies. Analysis of sections from patients with EBV-associated Hodgkin's lymphoma showed that most of the γδ T cells from these patients expressed Vγ9, whereas γδ T cells expressing Vγ2, 3 or 4 were nearly absent (Figure 5A). Furthermore, staining of cryosections obtained from patients with colon adenocarcinoma using the Vδ1 and Vγ2/3/4 antibodies revealed that these γδ T cells are enriched in respective malignancies, as shown for one representative patient (Figure 5B). This indicates that the Vγ2/3/4 clone 23D12 is also suitable for staining cryosections.

Concluding remarks

The variety of available reagents for the characterization of γδ T cells is, as the research field itself, dynamic and growing, but currently still very limited. Here, we outlined a framework for the phenotypic and functional characterization of human γδ T cells based on currently available reagents. We are certainly aware that the above-described antibodies and antibody combinations still have room for improvement. On the part of the manufacturers, a broader spectrum of fluorophore-antibody conjugates, preferably of smaller size to minimize potential steric hindrance problems, would be appreciated. One must also be aware that rare αβ T cells harbor a TCR trans-rearrangement and thus may express a Vγ rather than a Vβ element. Such αβ T cells stain with anti-Vγ antibodies as shown here for Vγ5, and this has been reported previously also for Vγ4

and Vγ9 [25, 28]. When characterizing γδ T cells in tissue samples the following issues should be taken into account. First, one should carefully select the enzymes used for tissue dissociation in order to avoid the loss of certain cell surface markers. Besides that, sample size and in some cases the low abundance of γδ T cells may be limiting factors. For γδ T cell immunomonitoring within tumor-infiltrating lymphocytes or tumor-ascites lymphocytes by flow cytometry, the additional use of an anti-CD45 mAb in a multicolor panel is recommended for precise analysis of the different γδ and αβ T cell subsets surrounded by many other tumor-associated immune cells and tumor cells (Oberg et al. this volume). Furthermore, the development of antibodies suitable for staining paraffin-embedded tissue sections, and antibodies compatible with the fluorescence microscopy-based MACSima imaging system or the mass cytometry-based Hyperion imaging system could take γδ T cell research to a new level, supporting the various promising attempts to exploit these remarkable cells for treating infectious and non-infectious diseases as well as malignancies.

Authorship

Conceptualization: NB, DW, HO, GP, SK, KS, KWH

Investigation: NB, DW, HO, JB, BW, CG, SS, CR, HH, CP, PN, SK, KS, KWH

Data curation - formal analysis: NB, DW, HO, JB, SS, CR, HH, PN, SK, KS, KWH

Writing - original draft: NB, DW, KS, KWH

Writing - review, editing, and revision: NB, DW, HO, GP, MWL, SK, DK, KS, KWH

Acknowledgments

The authors gratefully thank Janine Spreuer for technical help and Sandra Kröger for her support in staining the tissues of patients with EBV-associated Hodgkin's lymphoma. HH was supported by a grant from the Stefan-Morsch Foundation and KS was supported by a grant from the Jürgen Manchot Foundation. KWH received funding from the DFG (WI 5021-21 – FOR2799), the Klaus Tschira Foundation (00.316.2017) and the Medical Faculty of the University of Tübingen (2509-0-0). DW obtained funding from the DFG (WE 3559/6-1 – FOR2799), DK obtained funding from the DFG (Ka 502/19-1).

Conflict of Interest Disclosure

DK is a member of scientific advisory boards of Incysus, Imcheck, Lava Therapeutics, and Qu Biologics.GP has received research support from Immatics Biotechnologies GmbH, speaker's honoraria from Celgene, Pfizer, Sanofi, 4D-Pharma, Clasado, and Seqirus and is a Consultant to Repair Biotechnologies, Inc. KWH received commercial research grants from Catalym GmbH and travel support from SITC (Society for Immunotherapy of Cancer). MWL is an inventor of patents owned by Immatics

Biotechnologies GmbH. No potential conflicts of interest were disclosed by the other authors.

References

1. Chien, Y. H., Jores, R., Crowley, M. P. 1996 Recognition by gamma/delta T cells. Annual Review of Immunology 14, 511-532.
2. Vantourout, P. and Hayday, A. 2013 Six-of-the-best: unique contributions of gammadelta T cells to immunology. Nat Rev Immunol 13, 88-100.
3. Ramstead, A. G. and Jutila, M. A. 2012 Complex Role of gamma delta T-Cell-Derived Cytokines and Growth Factors in Cancer. J Interf Cytok Res 32, 563-569.
4. Couzi, L., Pitard, V., Sicard, X., Garrigue, I., Hawchar, O., Merville, P., Moreau, J. F., Dechanet-Merville, J. 2012 Antibody-dependent anti-cytomegalovirus activity of human gamma delta T cells expressing CD16 (Fc gamma RIIIa). Blood 119, 1418-1427.
5. Brandes, M., Willimann, K., Moser, B. 2005 Professional antigen-presentation function by human gamma delta T cells. Science 309, 264-268.
6. Wen, L., Pao, W., Wong, F. S., Peng, Q. S., Craft, J., Zheng, B., Kelsoe, G., Dianda, L., Owen, M. J., Hayday, A. C. 1996 Germinal center formation, immunoglobulin class switching, and autoantibody production driven by "Non alpha/beta" T cells. J Exp Med 183, 2271-2282.
7. Deusch, K., Luling, F., Reich, K., Classen, M., Wagner, H., Pfeffer, K. 1991 A Major Fraction of Human Intraepithelial Lymphocytes Simultaneously Expresses the Gamma/Delta T-Cell Receptor, the Cd8 Accessory Molecule and Preferentially Uses the V-Delta-1 Gene Segment. European Journal of Immunology 21, 1053-1059.
8. Davey, M. S., Willcox, C. R., Joyce, S. P., Ladell, K., Kasatskaya, S. A., McLaren, J. E., Hunter, S., Salim, M., Mohammed, F., Price, D. A., Chudakov, D. M., Willcox, B. E. 2017 Clonal selection in the human Vdelta1 T cell repertoire indicates gammadelta TCR-dependent adaptive immune surveillance. Nat Commun 8, 14760.
9. Davey, M. S., Willcox, C. R., Hunter, S., Kasatskaya, S. A., Remmerswaal, E. B. M., Salim, M., Mohammed, F., Bemelman, F. J., Chudakov, D. M., Oo, Y. H., Willcox, B. E.

2018 The human Vdelta2(+) T-cell compartment comprises distinct innate-like Vgamma9(+) and adaptive Vgamma9(-) subsets. Nat Commun 9, 1760.

10. Gentles, A. J., Newman, A. M., Liu, C. L., Bratman, S. V., Feng, W. G., Kim, D., Nair, V. S., Xu, Y., Khuong, A., Hoang, C. D., Diehn, M., West, R. B., Plevritis, S. K., Alizadeh, A. A. 2015 The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med 21, 938-945.

11. Oberg, H. H., Grage-Griebenow, E., Adam-Klages, S., Jerg, E., Peipp, M., Kellner, C., Petrick, D., Gonnermann, D., Freitag-Wolf, S., Rocken, C., Sebens, T., Vogel, I., Becker, T., Ebsen, M., Kabelitz, D., Wesch, D., Sebens, S. 2016 Monitoring and functional characterization of the lymphocytic compartment in pancreatic ductal adenocarcinoma patients. Pancreatology 16, 1069-1079.

12. Oberg, H. H., Peipp, M., Kellner, C., Sebens, S., Krause, S., Petrick, D., Adam-Klages, S., Rocken, C., Becker, T., Vogel, I., Weisner, D., Freitag-Wolf, S., Gramatzki, M., Kabelitz, D., Wesch, D. 2014 Novel bispecific antibodies increase gammadelta T-cell cytotoxicity against pancreatic cancer cells. Cancer Res 74, 1349-60.

13. Dechanet, J., Merville, P., Lim, A., Retiere, C., Pitard, V., Lafarge, X., Michelson, S., Meric, C., Hallet, M. M., Kourilsky, P., Potaux, L., Bonneville, M., Moreau, J. F. 1999 Implication of gammadelta T cells in the human immune response to cytomegalovirus. J Clin Invest 103, 1437-49.

14. Halary, F., Pitard, V., Dlubek, D., Krzysiek, R., de la Salle, H., Merville, P., Dromer, C., Emilie, D., Moreau, J. F., Dechanet-Merville, J. 2005 Shared reactivity of V delta 2(neg) gamma delta T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. J Exp Med 201, 1567-1578.

15. Mordmuller, B., Surat, G., Lagler, H., Chakravarty, S., Ishizuka, A. S., Lalremruata, A., Gmeiner, M., Campo, J. J., Esen, M., Ruben, A. J., Held, J., Calle, C. L., Mengue, J. B., Gebru, T., Ibanez, J., Sulyok, M., James, E. R., Billingsley, P. F., Kc, N., Manoj, A.,

Murshedkar, T., Gunasekera, A., Appen, A. G. E., Li, T., Stafford, R. E., Li, M. L., Felgner, P. L., Seder, R. A., Richie, T. L., Sim, B. K. L., Hoffman, S. L., Kremsner, P. G. 2017 Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature 542, 445-+.

16. Lawand, M., Dechanet-Merville, J., Dieu-Nosjean, M. C. 2017 Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. Front Immunol 8, 761.

17. Kabelitz, D., Ackermann, T., Hinz, T., Davodeau, F., Band, H., Bonneville, M., Janssen, O., Arden, B., Schondelmaier, S. 1994 New monoclonal antibody (23D12) recognizing three different V gamma elements of the human gamma delta T cell receptor. 23D12+ cells comprise a major subpopulation of gamma delta T cells in postnatal thymus. J Immunol 152, 3128-36.

18. Hinz, T., Wesch, D., Halary, F., Marx, S., Choudhary, A., Arden, B., Janssen, O., Bonneville, M., Kabelitz, D. 1997 Identification of the complete expressed human TCR V gamma repertoire by flow cytometry. Int Immunol 9, 1065-1072.

19. Kordasti, S., Costantini, B., Seidl, T., Perez Abellan, P., Martinez Llordella, M., McLornan, D., Diggins, K. E., Kulasekararaj, A., Benfatto, C., Feng, X., Smith, A., Mian, S. A., Melchiotti, R., de Rinaldis, E., Ellis, R., Petrov, N., Povoleri, G. A., Chung, S. S., Thomas, N. S., Farzaneh, F., Irish, J. M., Heck, S., Young, N. S., Marsh, J. C., Mufti, G. J. 2016 Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment. Blood 128, 1193-205.

20. Janssen, O., Wesselborg, S., Hecklostreicher, B., Pechhold, K., Bender, A., Schondelmaier, S., Moldenhauer, G., Kabelitz, D. 1991 T-Cell Receptor Cd3-Signaling Induces Death by Apoptosis in Human T-Cell Receptor Gamma-Delta+T-Cells. Journal of Immunology 146, 35-39.

21. Wesch, D., Beetz, S., Oberg, H. H., Marget, M., Krengel, K., Kabelitz, D. 2006 Direct costimulatory effect of TLR3 ligand poly(I : C) on human gamma delta T lymphocytes. Journal of Immunology 176, 1348-1354.

22. Correia, D. V., Fogli, M., Hudspeth, K., da Silva, M. G., Mavilio, D., Silva-Santos, B. 2011 Differentiation of human peripheral blood Vdelta1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. Blood 118, 992-1001.

23. Wistuba-Hamprecht, K., Pawelec, G., Derhovanessian, E. 2014 OMIP-020: Phenotypic characterization of human gammadelta T-cells by multicolor flow cytometry. Cytometry A 85, 522-4.

24. Zeng, X., Wei, Y. L., Huang, J., Newell, E. W., Yu, H., Kidd, B. A., Kuhns, M. S., Waters, R. W., Davis, M. M., Weaver, C. T., Chien, Y. H. 2012 Gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. Immunity 37, 524-34.

25. Davodeau, F., Peyrat, M. A., Gaschet, J., Hallet, M. M., Triebel, F., Vie, H., Kabelitz, D., Bonneville, M. 1994 Surface Expression of Functional T-Cell Receptor Chains Formed by Interlocus Recombination on Human T-Lymphocytes. J Exp Med 180, 1685-1691.

26. Soderstrom, K., Bucht, A., Halapi, E., Lundqvist, C., Gronberg, A., Nilsson, E., Orsini, D. L. M., Vandewal, Y., Koning, F., Hammarstrom, M. L., Kiessling, R. 1994 High Expression of V-Gamma-8 Is a Shared Feature of Human Gamma-Delta T-Cells in the Epithelium of the Gut and in the Inflamed Synovial Tissue. Journal of Immunology 152, 6017-6027.

27. Wesch, D., Hinz, T., Kabelitz, D. 1998 Analysis of the TCR V gamma repertoire in healthy donors and HIV-1-infected individuals. Int Immunol 10, 1067-1075.

516 28. Hinz, T., Allam, A., Wesch, D., Schindler, D., Kabelitz, D. 2000 Cell-surface expression of
517 transrearranged Vgamma-cbeta T-cell receptor chains in healthy donors and in ataxia
518 telangiectasia patients. Br J Haematol 109, 201-10.
519 29. Jungbluth, A. A., Frosina, D., Fayad, M., Pulitzer, M. P., Dogan, A., Busam, K. J., Imai,
520 N., Gnjatic, S. 2018 Immunohistochemical Detection of gamma/delta T Lymphocytes in
521 Formalin-fixed Paraffin-embedded Tissues. Appl Immunohistochem Mol Morphol.

522

Figure legends

Figure 1: Magnetic isolation of γδ T cells from PBMCs (A) Representative FACS plots showing the CD3 compartment pre and post isolation of untouched γδ T cell preparations. Presented data reflect the reproducibly high purities that are achieved using kits from either Miltenyi Biotec (Manufacturer M) or STEMCELL Technologies (Manufacturer S). Viability is reproducibly above 99%. **(B)** Representative FACS plots of cell isolates gained with positive selection strategy using kits from Miltenyi Biotec or STEMCELL Technologies. Shown are target cells in the $CD3^+$ gate. Viability is reproducibly above 98%. **(C)** $Vδ1^-Vδ2^-$ cell fractions from two healthy adult donors are presented (gated on $CD3^+$ T cells, no contaminating αβ T cells are present). Without the availability of an anti-human Vδ3 antibody these donors cannot be used for the isolation of negatively selected pure Vδ2 γδ T cells.

Figure 2: Phenotypic characterization of γδ T cells via flow cytometry and CyTOF **(A)** Phenotypic characterization of the Vδ1 and Vδ2 sub-populations via flow cytometry. Gating on peripheral, viable, $CD3^+$, lymphocytes showed that the γδ TCR antibody (clone 11F2) recognized all $Vδ1^+$ (clone REA173) and $Vδ2^+$ (clone 123R3) cells. Sufficient separation of the sub-populations, gated on γδ TCR^+ T cells, was achieved. **(B)** Direct and indirect staining of the γδ TCR with the clones 11F2 and IMMU510. The best separation with the lowest background signal in flow cytometry was achieved with the biotinylated 11F2 clone. The population was gated on viable, $CD3^+$ lymphocytes. **(C)** Characterization of γδ T cell sub-populations by CyTOF in a fixed and permeabilized representative sample. Both plots display the same population that was gated on viable,

546 $CD45^+$, $CD14^-$, $CD33^-$, $CD20^-$, $CD3^+$ T cells. γδ TCR (clone 11F2; Sm152) stained the
547 vast majority of the indirectly stained $Vδ1^+$ (clone TS8.2 FITC a Gd160), but none of the
548 $Vδ2^+$ (clone B6 ; Pr141) γδ T cells.

549

550 **Figure 3: Functional characterization of γδ T cells via flow cytometry. (A)** Analysis
551 of cytokine expression in fixed PBMC samples after stimulation with Zoledronate ($Vδ2^+$)
552 or PMA/Ionomycin ($Vδ1^+$). $Vδ1^+$ and $Vδ2^+$ cells were gated on viable, $CD3^+$, γδ TCR^+
553 lymphocytes. **(B)** Proliferation of $CD3^+γδTCR^-$, $Vδ1^+$ and $Vδ2^+$ T cells after stimulation
554 with PHA-L was tracked on the basis of dye dilution using CellTrace Violet. γδ T cell
555 subpopulations were gated on viable, $CD3^+$, γδ TCR^+ lymphocytes. The proliferation
556 modeling tool included in the FlowJo software enabled a more in-depth analysis of the
557 proliferative properties.

558

559 **Figure 4: Identification of Vγ3 and Vγ5 γδ and αβ T cell clones**. Viable cells were
560 discriminated by gating on lymphocytes (FSC vs. SSC) and by near infra-red live/dead-
561 staining. γδ and αβ T cell clones stained by mAb Vγ3/5 (clone 56.3) were co-labeled
562 with the mAbs recognizing the αβ TCR (clone IP26), γδ TCR (clone 11F2), Vγ2/3/4
563 (clone 23D12). (**A)** Vγ5 γδ clone ($IP26^-$,$11F2^+$,56.3^+,$23D12^-$) (**B)** Vγ3 γδ clone
564 (IP26,$11F2^+$,56.3^+,$23D12^+$); (**C)** Vγ5 αβ clone ($IP26^+$,$11F2^-$,56.3^+,$23D12^-$).

565

566 **Figure 5: Distribution of different T cell subsets in EBV-associated Hodgkin´s**
567 **lymphoma tissues and colon adenocarcinoma tissue**. **(A)** Serial paraffin-embedded

tissue sections from patients with EBV-associated Hodgkin´s lymphoma were stained with γδ TCR (clone γ3.20), Vγ9 (clone 7A5), Vγ2/3/4 (clone 23D12) mAbs as indicated in one representative donor. **(B)** Serial cryosections obtained from colon adenocarcinoma patients were stained with IgG control, Vδ1 (clone R3.12), Vγ2/3/4 (clone 23D12) mAbs as indicated in one representative patient. IHC staining was performed as described in the Materials and Methods section.

Table 1: Monoclonal antibody panels for the functional characterization and for tracking the proliferation of γδ T cells via flow cytometry

Specificity	Fluorophore	Clone
Functional characterization		
Dead cells	Fixable red	
CD3	BV510	UCHT1
γδ TCR	Biotin + Streptavidin-PE	11F2
Vδ1	PerCP-Vio700	REA173
Vδ2	FITC	123R3
IFN-γ	PE-Cy7	B27
TNF-α	Alexa Fluor 700	Mab11
IL17A	BV711	BL168
CD107a	Pacific Blue	H4A3
Tracking of proliferation		
Dead cells	Fixable red	
CD3	Alexa Fluor 700	UCHT1
γδ TCR	Biotin + Streptavidin-PE	11F2
Vδ1	APC	REA173
Vδ2	FITC	123R3

Figure 1

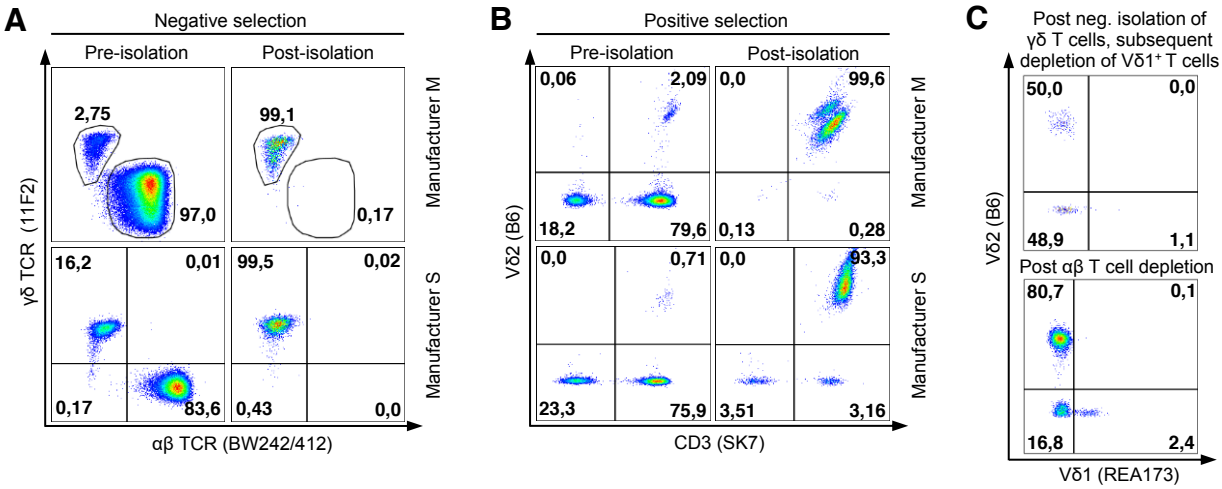

A
Negative selection
Pre-isolation
Post-isolation
2,75
97,0
99,1
0,17
16,2
0,01
99,5
0,02
0,17
83,6
0,43
0,0
γδ TCR (11F2)
αβ TCR (BW242/412)
Manufacturer M
Manufacturer S
B
Positive selection
Pre-isolation
Post-isolation
0,06
2,09
0,0
99,6
18,2
79,6
0,13
0,28
0,0
0,71
0,0
93,3
23,3
75,9
3,51
3,16
Vδ2 (B6)
CD3 (SK7)
Manufacturer M
Manufacturer S
C
Post neg. isolation of γδ T cells, subsequent depletion of Vδ1+ T cells
50,0
0,0
48,9
1,1
Post αβ T cell depletion
80,7
0,1
16,8
2,4
Vδ2 (B6)
Vδ1 (REA173)

Figure 2

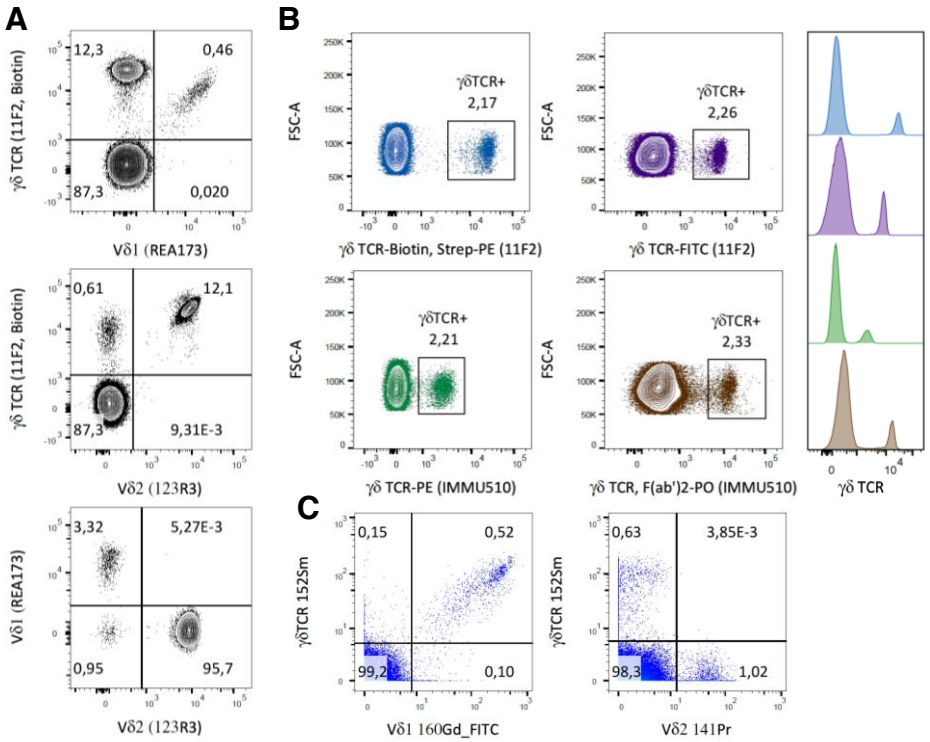

A
12,3
0,46
87,3
0,020
γδ TCR (11F2, Biotin)
Vδ1 (REA173)
0,61
12,1
87,3
9,31E-3
Vδ2 (123R3)
3,32
5,27E-3
0,95
95,7
Vδ1 (REA173)
Vδ2 (123R3)
B
γδTCR+
2,17
FSC-A
γδ TCR-Biotin, Strep-PE (11F2)
γδTCR+
2,26
γδ TCR-FITC (11F2)
γδTCR+
2,21
γδ TCR-PE (IMMU510)
γδTCR+
2,33
γδ TCR, F(ab')2-PO (IMMU510)
γδ TCR
C
0,15
0,52
99,2
0,10
γδTCR 152Sm
Vδ1 160Gd_FITC
0,63
3,85E-3
98,3
1,02
Vδ2 141Pr

Figure 3

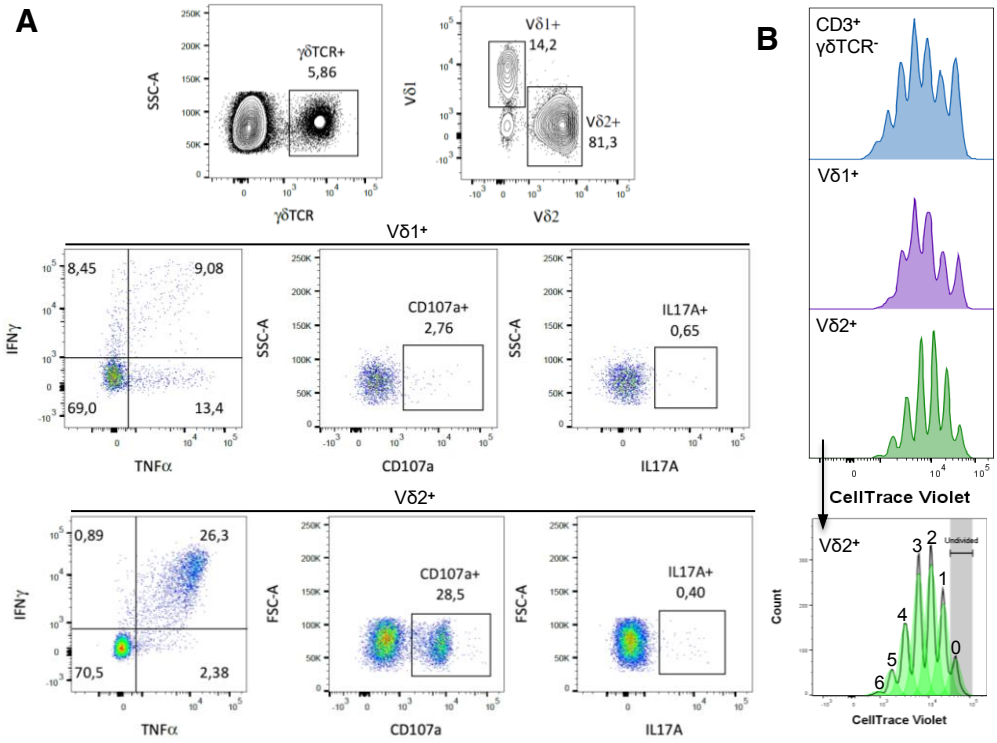

A
γδTCR+
5,86
SSC-A
γδTCR
Vδ1+
14,2
Vδ2+
81,3
Vδ1
Vδ2
Vδ1+
8,45
9,08
69,0
13,4
IFNγ
TNFα
CD107a+
2,76
SSC-A
CD107a
IL17A+
0,65
IL17A
Vδ2+
0,89
26,3
70,5
2,38
IFNγ
TNFα
CD107a+
28,5
FSC-A
CD107a
IL17A+
0,40
IL17A
B
CD3+
γδTCR-
Vδ1+
Vδ2+
CellTrace Violet
Vδ2+
Undivided
3
2
1
4
0
5
6
Count
CellTrace Violet

Figure 4

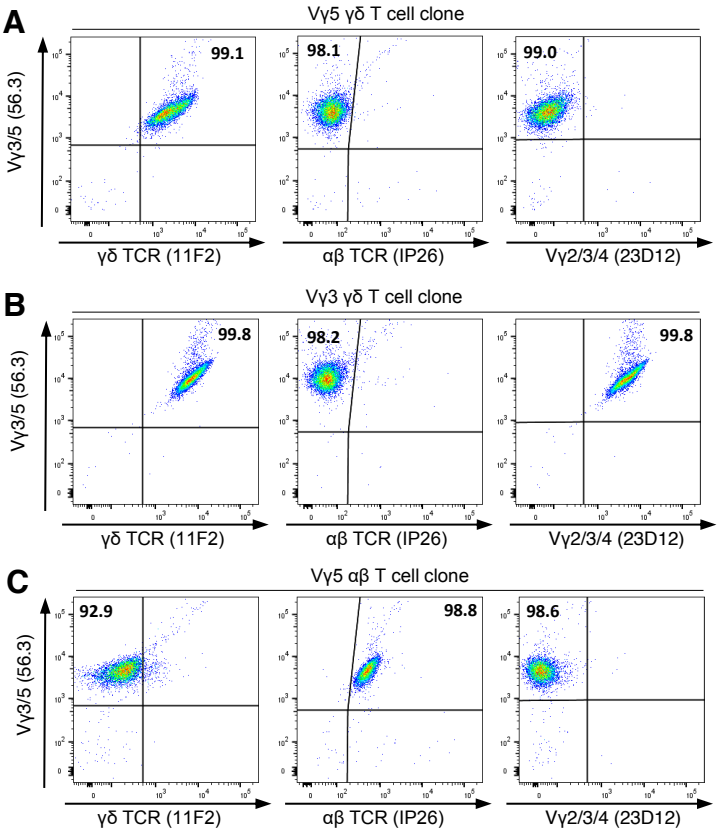
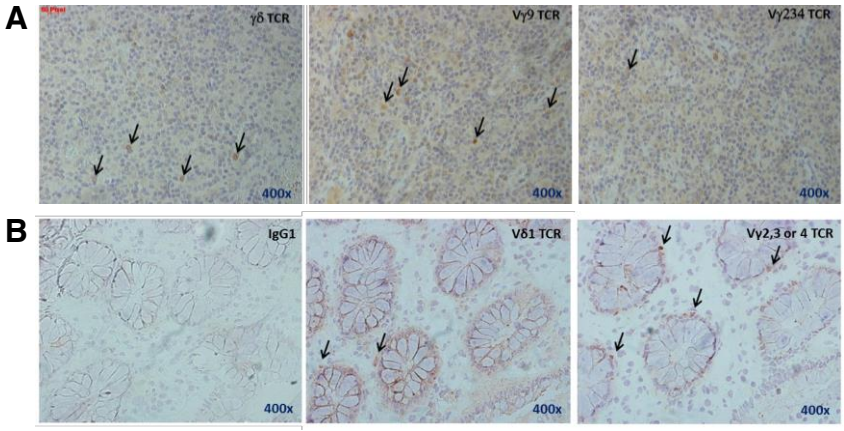

A
Vγ5 γδ T cell clone
99.1
98.1
99.0
Vγ3/5 (56.3)
γδ TCR (11F2)
αβ TCR (IP26)
Vγ2/3/4 (23D12)
B
Vγ3 γδ T cell clone
99.8
98.2
99.8
Vγ3/5 (56.3)
γδ TCR (11F2)
αβ TCR (IP26)
Vγ2/3/4 (23D12)
C
Vγ5 αβ T cell clone
92.9
98.8
98.6
Vγ3/5 (56.3)
γδ TCR (11F2)
αβ TCR (IP26)
Vγ2/3/4 (23D12)

Figure 5


A
γδ TCR
400x
Vγ9 TCR
400x
Vγ234 TCR
400x
B
IgG1
400x
Vδ1 TCR
400x
Vγ2,3 or 4 TCR
400x